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13. ABSTRACT *(Maximum 200*

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Our goal is to identify and characterize a breast cancer suppressor gene on 11p15. Indirect evidence suggests that this gene is lost in half of breast cancers that metastasize and thus plays a specific role in breast cancer metastasis. We have defined the region of chromosome loss in tumors, and we are identifying candidate genes within this region and analyzing them for mutations in breast cancer, using several approaches. We have identified three separate candidate genes within this region. These include NUP98, which shows a rare sequence variation in the germline of a breast cancer patient, not seen in 200 normal control patients. A second gene we identified is a novel homologue of Rb-associated protein 48. A third gene is a novel homologue of a gene involved in fas and fasL-mediated apoptosis, which is defective in breast cancer. We have also identified a novel mechanism by which breast cancers generate and undergo abnormal gene expression, namely aberrant splicing in the absence of mutations in the target genes themselves.

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Andrew Feeley 11/5/97

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

This project is based on the hypothesis that 11p15 contains at least one tumor suppressor gene involved in metastatic breast cancer (1-3), and our own data that loss of heterozygosity (LOH) of 11p15 occurs in 40% of metastatic breast cancers. Our overall goal is to localize and identify candidate 11p15 breast cancer genes. Please note that the originally proposed Task 5, loss of imprinting (LOI) in breast cancer, was deleted from the proposal with approval from the Army, because the initial funding was significantly below the requested and recommended level. A supplementary request in response to an RFA from the National Action Plan on Breast Cancer (NAPBC) was requested to restore those studies. It received an outstanding peer review priority rating but was not funded.

BODY OF REPORT

1. EXPERIMENTAL METHODS

Isolation of DNA and RNA from Tissues: Breast cancers and their matched normal tissues were obtained from The Johns Hopkins Hospital Pathology Department and the Cooperative Human Tissue Network, and normal fetal tissues were from the University of Washington Fetal Tissue Bank. The tissues were stored at -135°C until use. Breast cancers represented stages 2-4. The tissues were pulverized in liquid nitrogen and suspended in TE9 [0.5 M Tris-HC1 (pH 9.0), 20 mM EDTA (pH 8.0), and 10 mM NaC1]. Proteinase K (0.2 mg/ml) and 1% SDS were added to lyse the cells and digest the proteins at 50°C for overnight. To isolate RNA, tissues were cut into small pieces and homogenized in 4 ml of RNAzol B (Tel-Test). RNAs were stored at -70°C.

PCR and Southern and Northern Hybridization: Five μg of genomic DNA was digested with 10 units of restriction enzyme at 37°C overnight. Digested DNAs were resolved on 0.8% agarose gel, transferred to Hybond-N^+ filters, and fixed by UV cross-linking. Probes were labeled by the random priming method (4). Hybridizations were carried out at 65°C overnight in Church-Gilbert buffer [0.5 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM EDTA] (5). Northern hybridization was performed as described (6).

RNase Protection Assay: PCR-generated gene fragments were subcloned into pBluescript II KS +/- (Stratagene) for generation of antisense RNA probes, using the MAXIscript in vitro transcription kit (Ambion) and a-^{32}P-UTP according to the manufacturer's directions. 1 μg of pre-purified pTRI-GAPDH (Ambion) was also used to generate antisense GAPDH transcripts for quantitation controls. RNase protection assays were carried out utilizing the RPA II Kit (Ambion) with approximately 20 μl of poly(A+) mRNA per reaction.

RT-PCR for Aberrant Splicing of TSG101: 2 mg RNA was mixed with 50 ng primer 1 in 35 ml DEPC water, denatured at 70°C for 10 min, and the reverse transcription reaction was carried out in 50 ml solution containing 1X buffer (10 mM Tris, pH8.0/50 mM KCl/1.5 mM MgCl), 0.2 mM dNTP, 1 unit/μl RNase inhibitor, and 3 units AMV reverse transcriptase. The reaction mixture was incubated at 42°C for 1 hour and cDNA was stored at -20°C. RT-PCR reactions were carried out as described (7). The RT-PCR products were analyzed in 1.2%

agarose gels. DNA fragments were cut out and purified with Qiaex II (Qiagen). DNAs were directly sequenced using an ABI377 automatic sequencer. To quantify the PCR products, we labelled the primer 4 with T4 polynucleotide kinase and used 5' end labelled primer 4 in the PCR reaction. Agarose gels were dried and analyzed by Phosphorimager (Molecular Dynamics). The primers used for PCR and sequencing were as follows: primer 1, ATTTAGCAG-TCCCAACATTCAGCACAAA; primer 2, CGGGTGTCGGAGAGCCAGCTCAAGAAA; primer 3, CCTCCAGCTGGTATCAGAGAAGTCGT; primer 4, AGCCAGCTCAAG-AAAATGGTG TCCAAG; primer 5, TCACTGAGACCGGCAGTCTTTCTTGCTT; primer 6, TTGTCACTGACCGCAGAG; primer 7, ATAGGATGCCGAAATAGG; primer 8, CCATTCATGTAGATAAGG.

Microcell Transfer of Subchromosomal Transferable Fragments (STF's): STF's were prepared as described (8). MCF-7 and HuMI cells were cultured in DMEM/10% fetal calf serum. A9 cells carrying STF's were cultured for 48 hours in cytochalasin B and G418 (400 μg/ml). Microcells were prepared by culturing STF-containing donor cells in colcemid (0.5 μg/ml) for 48 hours, centrifuging in cytochalasin B (10 μg/ml), and filtering, and then fused to recipient tumor cells with PEG as described (9).

Isolation of Genomic Clones and Physical Mapping: STS's previously isolated and/or mapped to 11p15.5 (10) were more precisely localized within STF 74-1-6 by the presence of PCR amplification, and as a negative control, absence of amplification from its parental cell A9. BAC (Genetic Research, Inc.) and PAC (Genome System, Inc.) libraries were screened for the presence of STS's by PCR. P1 clones (Genome Systems, Inc.) were screened by hybridization of a high density filter using genomic fragment as probes. Cosmids derived from YAC's B40E4, D122D10, and E42F4 were described previously (10). Cosmids derived from YAC B176E9 and B215A11 were isolated by constructing cosmid libraries using total YAC DNA. Assembly of genomic contigs using these BAC, PAC, and P1 clones was achieved by PCR using STS, end clones, and exons.

Exon Trapping: Exon trapping was performed using the pSPL3B or pSPL3B-CAM vectors (LTI). Individual BAC clones were double-digested with Bam HI and Bgl II and subcloned into the Bam HI site of splicing vector pSPL3B. PAC, P1, or pools of 4 cosmids were subcloned in pSPL3B-CAM. Plasmid DNAs were isolated from 20 randomly chosen transformants and analyzed by restriction digestion for the presence of inserts. If more than 50% of transformants contained inserts of differing sizes and the total number of transformants exceeded 1000, LB medium was added to the plates containing transformants and colonies were pooled for isolation of DNA. Aliquots of the transformation mixtures were saved as bacterial stocks. DNA was transfected into Cos 7 cells by lipofection. Cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Confluent cells were split 1:3 one day prior to transfection. RNA was isolated 24 hours post-transfection using RNAzol B (Tel-Test) according to the manufacturer's protocol. RT-PCR was used to amplify exons using primer set SD2-SA4 provided in the exon trapping kit and subcloned in the pAMP10 vector (LTI). The pAMP10 subclones were screened by PCR using primer set SD2-SA4. Transformants with PCR products larger than 250 bp were selected, and they corresponded to exons with sizes above 73 bp. DNAs were isolated from these clones containing exons using the Wizard miniprep kit (Promega) and exons were sequenced using the primer SD2.

cDNA Isolation and Sequence Analysis: cDNAs were isolated by either of three methods. First, we have developed a novel PCR-based cDNA screening method in order to isolate cDNAs directly and systematically containing the exons (11). Second, the GenBank dbEST and TIGR THC databases were searched for ESTs using exon sequences. Third, cDNA libraries were screened by hybridization using probes prepared by random priming (4). Sequence analysis of both exons and cDNAs were carried out using Sequencher, GCG, and BLAST (12) software.

2. RESULTS

Task 1, LOH Mapping of 11p15: We have mapped LOH of 11p15 in breast cancer by analyzing 72 primary tumors from stages 2-4 using a panel of 12 highly informative polymorphic markers from 11p15. The highest fraction of LOH centered at D11S860 (40%). These data are illustrated in Figure 1. LOH at a frequency > 10% extended at the proximal end to D11S988 and at the distal end to D11S1318, although there may be a second more telomeric locus. The smallest interval to which we can currently localize a breast tumor suppressor gene is a region consistent with these frequencies, based on individual tumors with LOH, using a stringent scoring system. The centromeric boundary of the region is between D11S860 and D11S4146, and the telomeric boundary is between D11S1318 and TH. In the past year, we have also 20 new highly informative markers within this region, and we will re-examine tumors that did not show LOH for evidence of interstitial deletion within this domain.

Task 2, Functional Localization of an 11p15 Gene: We were able to overcome the initial technical problem of recipient MCF-7 cells undergoing in vitro growth arrest. We found that this was cell density dependent, and that when cells were replated at high density after microcell transfer, we could propagate them stably in vitro. However, we encountered a new problem in the past year, that we also fell we have recently overcome. We found that MCF-7 cells variably acquired tumorigenicity after introduction into nude mice, even when containing

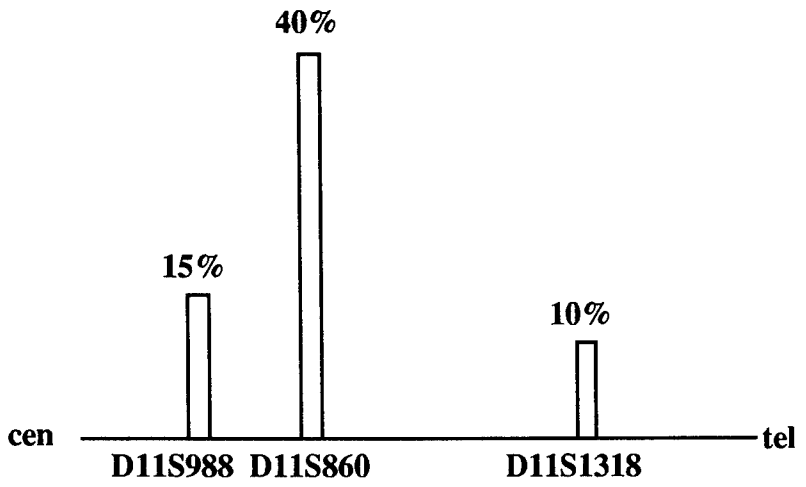

Figure 1. Frequency of LOH in breast cancer. 72 primary breast cancers were analyzed.

tumor-suppressing subchromosomal transferable fragment 74-1-6. We have had the same problem with the cell line G401, and it appears that expression of the suppressor gene in this fragment is not irreversible under the powerful growth of the selective conditions of nude mouse tumorigenicity, and that it can undergo variable silencing, which is then selected for in the mice. We have not had this problem with RD cells, however. We believe that we can overcome this problem by using another STF, 74-2, since this problem seems to be unique to the STF we previously used, as 74-2 does not appear to lose tumorigenicity in G401 cells.

Despite the difficulties in that area, we have made an exciting new discovery in the past year of a novel type of genetic abnormality in breast cancer, namely aberrant splicing in the absence of mutation due to relaxation of splicing fidelity rather than mutations in the target genes theirselves. This work has received considerable attention and led to a high priority "Advances in Brief" report in Cancer Research in the current funding year (13).

Tasks 3 and 4, Identification and Characterization of Candidate Breast Cancer Genes: This work has been highly productive in the last year. We have now identified several candidate genes within the interval identified in Task 1, and we are screening these genes for mutations in breast cancer. The first of these genes, as described earlier, is the nucleoporin gene NUP98. We and our collaborators previously reported the identification of a gene fusion between NUP98 and HOXA9, in patients with acute myeloid leukemia showing the translocation t(7;11)(p15;p15) (14). Northern analysis revealed both 4 kb and 7 kb isoforms of the gene. We previously reported the 4 kb sequence (14), but we have now isolated cDNA clone of NUP98 containing a 6.5 kb insert, corresponding to the 7 kb isoform of the gene. We have also confirmed that the 7 kb isoform of the NUP98 was generated by alternative RNA splicing.

Because of the proximity of NUP98 to D11S860 (< 200 kb), its known role in nuclear transport (15), and a reported defect of nuclear translocation in the familial breast cancer gene BRCA1 (16), we have analyzed NUP98 for mutations in breast cancer. We have now studied the entire coding region in 24 primary breast tumors by DNA sequencing, which is a more sensitive assay than single-strand conformational polymorphism (SSCP)-PCR, which we had used previously. One sequence variation was identified, causing a Thr85 to Ala85 substitution. However, normal tissue was heterozygous for this variant, indicating that the mutation was present in the germline. Nevertheless, 200 unrelated individuals did not show the substitution, and thus rare mutations in NUP98 may predispose to the development of breast cancer.

A second gene, that we isolated in the current year, shows homology to a potentially important cancer-related gene. We initially identified 2 EST's, homologous to exons we had trapped and located immediately centromeric to NUP98. We sequenced these two cDNA clones, which were found to overlap. We then identified a third exon that RT-PCR indicated is part of the same gene, which we term TSSC1. The complete sequence of this gene reveals that it encodes a predicted protein of 387 amino acids (Fig. 2) and that it shows homology to tumor suppressor RB-associated protein p48 (RBAP48) (ref. 17) ($p = 10^{-10}$) and to Drosophila chromatin assembly factor 1 (ref. 18) ($p=10^{-10}$). The sequence of this novel gene is shown in Figure 2. We are currently analyzing this gene for potential mutations in breast cancer. However, its homology to a gene associated with the RB tumor suppressor gene, as well as to the CAF-1 gene, suggest a potential role in breast cancer.

```
AATTCGGCACGAGAAGACTTCCAGTTTGGAGTCGTTTGCTGCGGGGAGGGAATGAATGGG
CGCTGGGAACACGCCCGCGAGGTGGGGACGCGCCGGCCGTAGCGAGGTCCTTAGCGTGTG
AGTGGCCGGGGTCGGGTCGCTTCCCCGCAGCATGGAGGACGATGCACCAGTGATCTACGG
                                    M  E  D  D  A  P  V  I  Y  G
GCTGGAGTTCCAGGCACGTGCCTTAACACCTCAAACTGCAGAAACAGATGCCATTCGGTT
  L  E  F  Q  A  R  A  L  T  P  Q  T  A  E  T  D  A  I  R  F
TTTGGTTGGGACGCAGTCTCTTAAATATGATAATCAGATCCATATCATAGATTTTGACGA
  L  V  G  T  Q  S  L  K  Y  D  N  Q  I  H  I  I  D  F  D  D
TGAAAACAACATTATAAATAAAAATGTCCTCCTCCATCAAGCGGGTGAAATCTGGCATAT
  E  N  N  I  I  N  K  N  V  L  L  H  Q  A  G  E  I  W  H  I
TAGCGCTAGCCCTGCAGACAGAGGTGTGCTGACGACCTGCTACAACAGAACTTCAGACAG
  S  A  S  P  A  D  R  G  V  L  T  T  C  Y  N  R  T  S  D  S
CAAAGTCCTGACATGTGCAGCCGTGTGGAGGATGCCGAAGGAATTGGAATCAGGCAGCCA
  K  V  L  T  C  A  A  V  W  R  M  P  K  E  L  E  S  G  S  H
CGAGTCCCCTGATGATTCATCCAGCACTGCACAGACCCTGGAGCTGCTCTGTCACCTTGA
  E  S  P  D  D  S  S  S  T  A  Q  T  L  E  L  L  C  H  L  D
CAACACAGCCCATGGCAACATGGCCTGTGTCGTGTGGGAGCCAATGGGAGATGGGAAGAA
  N  T  A  H  G  N  M  A  C  V  V  W  E  P  M  G  D  G  K  K
AATCATTTCCTTGGCTGATAACCATATCCTGCTGTGGGATTTACAGGAAAGCTCGAGCCA
  I  I  S  L  A  D  N  H  I  L  L  W  D  L  Q  E  S  S  S  Q
GGCTGTGCTGGCCAGCTCAGCGTCCCTGGAAGGGAAGGGACAACTGAAGTTCACCTCAGG
  A  V  L  A  S  S  A  S  L  E  G  K  G  Q  L  K  F  T  S  G
ACGGTGGAGCCCACATCATAACTGCACCCAGGTGGCCACAGCGAACGACACCACCCTCCG
  R  W  S  P  H  H  N  C  T  Q  V  A  T  A  N  D  T  T  L  R
TGGCTGGGACACCCGGAGCATGAGCCAGATCTACTGCATAGAGAATGCCCACGGACAGCT
  G  W  D  T  R  S  M  S  Q  I  Y  C  I  E  N  A  H  G  Q  L
GGTGCGGGACCTTGACTTTAATCCCAATAAGCAGTACTACTTGGCCAGCTGCGGAGACGA
  V  R  D  L  D  F  N  P  N  K  Q  Y  Y  L  A  S  C  G  D  D
CTGTAAGGTGAAGTTCTGGGACACCCGAAATGTCACCGAACCCGTGAAGACCCTGGAGGA
  C  K  V  K  F  W  D  T  R  N  V  T  E  P  V  K  T  L  E  E
GCACTCCCACTGGGTGTGGAACGTCCGCTACAACCACTCTCATGACCAGCTGGTCCTCAC
  H  S  H  W  V  W  N  V  R  Y  N  H  S  H  D  Q  L  V  L  T
GGGCAGCAGTGACAGCAGAGTCATCCTTTCCAACATGGTGTCCATCTCGTCGGAGCCCTT
  G  S  S  D  S  R  V  I  L  S  N  M  V  S  I  S  S  E  P  F
CGGCCACTTGGTAGACGACGATGACATCAGTGACCAGGAGGACCACCGTTCTGAAGAGAA
  G  H  L  V  D  D  D  D  I  S  D  Q  E  D  H  R  S  E  E  K
GAGCAAGGAGCCCCTGCAGGACAACGTGATCGCCACCTACGAGGAGCACGAGGACAGCGT
  S  K  E  P  L  Q  D  N  V  I  A  T  Y  E  E  H  E  D  S  V
CTATGCCGTGGACTGGTCCTCGGCTGACCCGTGGCTGTTTGCCTCCCTGAGCTATGACGG
  Y  A  V  D  W  S  S  A  D  P  W  L  F  A  S  L  S  Y  D  G
GAGGCTCGTGATCAACAGGGTGCCCAGGGCCCTGAAGTACCACATCCTGCTATGACTCCC
  R  L  V  I  N  R  V  P  R  A  L  K  Y  H  I  L  L  *
GGGCCTGGGTTATCCAGGTCCCATTGAGTGGTTTTCCTCTTGGCAGATTCTCAAACAGTC
GCAGCTCTTTGGAGGTGACTCGTGTTCCAGGTGGATCCCTCTCTGGGAGAGCCGCTGTTC
CCTTCCTGTAGCAGCAGCATTTATGAATGGGGTGAATGGGGCTATTGTCGACGGCACAGC
TAATGCCCGAACCCAGCCCCTGTCGGCAGAGACAGAGCCCCACATTATTATGTGAATAAC
AATGTTTTCTGTTTTAAGGGTGTCAGGAGTTTCGCTTTTTAAAAAAATGTCTGTTCCTGC
AGTAGTAACTCTTCTTTCTCTTGAGAGTAAAAAATGAAATAAAATAAATCCACGCTGACA
AAAAAAAAAAAAAAAAAAAAAAAAA
```

2A

Figure 2. Sequence of TSSC1 and homology to RbAp48. A. Nucleotide and predicted amino acid sequences of TSSC1. The cDNA of TSSC1 contained 1705 bp. The initiation methionine is at nucleotide 152, and the stop codon is at nucleotide 1315. The open reading frame contains 387 amino acids. Underlined sequences indicate three exon-trapped sequences. One exon is from 808 to 972, and the other exon is from 973 to 1140. B. (Next page) Comparison of amino acid sequences of TSSC1 and RbAp48 (shown) and RbAp46 (CAF1, not shown). + denotes conservative amino acid substitutions. TSSC1 shows 30% identity to RbAp48, and the homology is more extensive in the C-terminal region. Comparison was done using BLASTP.

```
TSSC1    142 WEPMGDGKKIISLADNHILLWD 163
             W P   G  + +  D+ I LWD
RbAp48   185 WNPNLSGHLLSASDDHTICLWD 206

TSSC1    192 WSPHHNCTQVATANDTTLRGWDTRS 216
             W   H     + A+D  L  WDTRS
RbAp48   235 WHLLHESLFGSVADDQKLMIWDTRS 259

TSSC1    225 NAHGQLVRDLDFNPNKQYYLASCGDDCKVKFWDTRNVTEPVKTLEEHSHWVWNVRYNHSHDQLVLTGSSDSRVILSNMVSISSE 308
             +AH   V  L FNP  ++ LA+   D  V  WD RN+   + + E H   ++ V+++  ++ ++ +  +D R+ + ++  I  E
RbAp48   270 DAHTAEVNCLSFNPYSEFILATGSADKTVALWDLRNLKLKLHSFESHKDEIFQVQWSPHNETILASSGTDRRLNVWDLSKIGEE 353

TSSC1    346 HEDSVYAVDWSSADPWLFASLSYD 369
             H   +    W+  +PW+  S+S D
RbAp48   373 HTAKISDFSWNPNEPWVICSVSED 396
```

2B

A third cDNA, which we term TSSC3, that we identified in the current year, is also a promising candidate for a breast cancer gene. We identified an EST corresponding to an exon we earlier trapped from genomic DNA. An additional BLAST search identified a second EST, which extended the sequence to the 5' end. Sequence analysis revealed that TSSC2 encodes a predicted protein of 152 amino acids, and that it shows strong homology to mouse TDAG51 ($p=10^{-32}$). The sequence of this gene is shown in Figure 3. Mouse TDAG51 upregulates fas and fasL, and thereby causes apoptosis of T-cell hybridoma cells (19).

```
CCCGCGCTCGGCACGACATGAAATCCCCCGACGAGGTGCTACGCGAGGGCGAGTTGGAGA
                    M  K  S  P  D  E  V  L  R  E  G  E  L  E
AGCGCAGCGACAGCCTCTTCCAGCTATGGAAGAAGAAGCGCGGGGTGCTCACCTCCGACC
K  R  S  D  S  L  F  Q  L  W  K  K  K  R  G  V  L  T  S  D
GCCTGAGCCTGTTCCCCGCCAGCCCCCGCGCGCGCCCCAAGGAGCTGCGCTTCCACTCCA
R  L  S  L  F  P  A  S  P  R  A  R  P  K  E  L  R  F  H  S
TCCTCAAGGTGGACTGCGTGGAGCGCACGGGCAAGTACGTGTACTTCACCATCGTCACCA
I  L  K  V  D  C  V  E  R  T  G  K  Y  V  Y  F  T  I  V  T
CCGACCACAAGGAGATCGACTTCCGCTGCGCGGGCGAGAGCTGCTGGAACGCGGCCATCG
T  D  H  K  E  I  D  F  R  C  A  G  E  S  C  W  N  A  A  I
CGCTGGCGCTCATCGATTTCCAGAACCGCCGCGCCCTGCAGGACTTTCGCAGCCGCCAGG
A  L  A  L  I  D  F  Q  N  R  R  A  L  Q  D  F  R  S  R  Q
AACGCACCGCACCCGCCGCACCCGCCGAGGACGCCGTGGCTGCCGCGGCCGCCGCACCCT
E  R  T  A  P  A  A  P  A  E  D  A  V  A  A  A  A  A  A  P
CCGAGCCCTCGGAGCCCTCCAGGCCATCCCCGCAGCCCAAACCCCGCACGCCATGAGCCC
S  E  P  S  E  P  S  R  P  S  P  Q  P  K  P  R  T  P  *
GCCGCGGGCCATACGCTGGACGAGTCGGACCGAGGCTAGGACGTGGCCGGCGCTCTCCAG
CCCTGCAGCAGAAGAACTTCCCGTGCGCGCGGATCCTCGCTCCGTTGCACGGGCGCCTTA
AGTTATTGGACTATCTAATATCTATGTATTTATTTCGCTGGTTCTTTGTAGTCACATATT
TTATAGTCTTAATATCTTGTTTTTGCATCACTGTGCCCATTGCAAATAAATCACTTGGCC
AGTTTGCTTTTCTAAAAAAAAAAAAAAAAAAAAA
```

3A

Figure 3. Sequence of TSSC3 and homology to mouse TDAG51. A. Nucleotide and predicted amino acid sequences of TSSC3. The cDNA of TSSC3 contains 754 bp. The initiation methionine starts is at nucleotide 476, and the stop codon is at nucleotide 476. The open reading frame encodes 152 amino acids. Underlined nucleotides indicate the trapped exon 3952 sequence. B. (next page) Comparison of amino acid sequences of TSSC3 and mouse TDAG51. + denotes conservative amino acid substitutions. TSSC3 shows 55% identity to mouse TDAG51. Comparison was done using BLASTP.

```
TSSC3       6 EVLREGELEKRSDSLFQLWKKKRGVLTSDRLSLFP 40
              + L+EG LEKRSD L QLWKKK  +LT + L L P
TDAG51      8 KALKEGVLEKRSDGLLQLWKKKCCILTEEGLLLIP 42

TSSC3      43 PRARPKELRFHSILKVDCVERTGKYVYFTIVTTDHKEIDFRCAGESCWNAAIALALIDFQNRRAL 107
              P  + KEL F ++  VDCVER GKY+YFT+V T+ KEIDFRC  +  WNA I L ++ ++NR+A+
TDAG51     75 PPVKLKELHFSNMKTVDCVERKGKYMYFTVVMTEGKEIDFRCPQDQGWNAEITLQMVQYKNRQAI 139
```

3B

What is most intriguing about a potential role for this gene in breast cancer is that breast cancer lines are defective in fas-mediated apoptosis and fas expression (20). Thus, TSSC3 must be regarded as an attractive candidate tumor suppressor gene for breast cancer. An additional gene that has attracted considerable interest is TSG101, which is described under Task 7 below.

Task 6, Analysis of Genes as Markers of Disease Subtype: This task involves statistical analysis of disease stage and specific alterations in 11p15 genes in breast cancer. The most frequent observed alteration to date, aberrant splicing of TSG101, was seen in all stages of disease. Although the frequency of this alteration is high (42%), only 12 samples have been analyzed so far. We will perform statistical analysis on the association of disease stage of this novel type of alteration on larger numbers of samples, both at this gene and other genes described under new Task 7, as well as on the association of disease stage with mutations of other alterations of the candidate genes we have identified and are identifying.

Task 7 (New), Aberrant Splicing of TSG101 in Human Breast Cancer: During the past year, another laboratory claimed to have cloned one of the genes that this grant was designed to identify. Thus, it was imperative for us to determine whether or not that claim was correct, so that we would not unnecessarily duplicate effort. That gene, TSG101, was initially isolated by random homozygous knockout, selecting for inactivated tumor suppressor genes using the NIH3T3 cell transformation system (21). The other laboratory then isolated the human clone and mapped it to 11p15 (7). They claimed that this gene, the human homologue of TSG101, maps to the region of LOH of 11p15 in breast cancer (7). They also claimed to observe frequent mutations of TSG101 in breast cancer, involving intragenic deletions. We thus undertook to prove or disprove this assertion by the other laboratory. As discussed below, we disproved their assertion that this is an 11p15 breast cancer suppressor gene. However in the course of these experiments, we discovered a novel type of genetic alteration in breast cancer, namely aberrant splicing of genes in the absence of mutations in the target genes themselves.

We first mapped the TSG101 gene using the STF's developed under Task 2. TSG101 clearly did not map within the tumor-suppressing STF's. Further experiments using somatic cell hybrids with different portions of chromosome 11 showed that the gene maps centromeric to

these STF's. Thus, our preliminary data suggested that TSG101 is not the gene that maps to the maximal region of LOH in breast cancer.

We then performed Southern hybridization to test directly the hypothesis that TSG101 undergoes intragenic deletions in breast cancer. We performed genomic Southern blots of 72 primary breast tumors with the TSG101 cDNA. As shown in Figure 4, tumors did not show any deletions or rearrangements by Southern blotting, that could explain the truncated transcripts described by the other laboratory. The restriction digestion patterns were identical for all tumors, matched normal tissues, and unrelated normal tissues. We examined 72 primary tumors, digesting DNA with Bgl II, Pst I, Pvu II, Eco RI, Bam HI, and Sal I. The lack of any alteration on Southern blots indicated that the truncated transcripts were not caused by intragenic deletions of sufficient size to account for them.

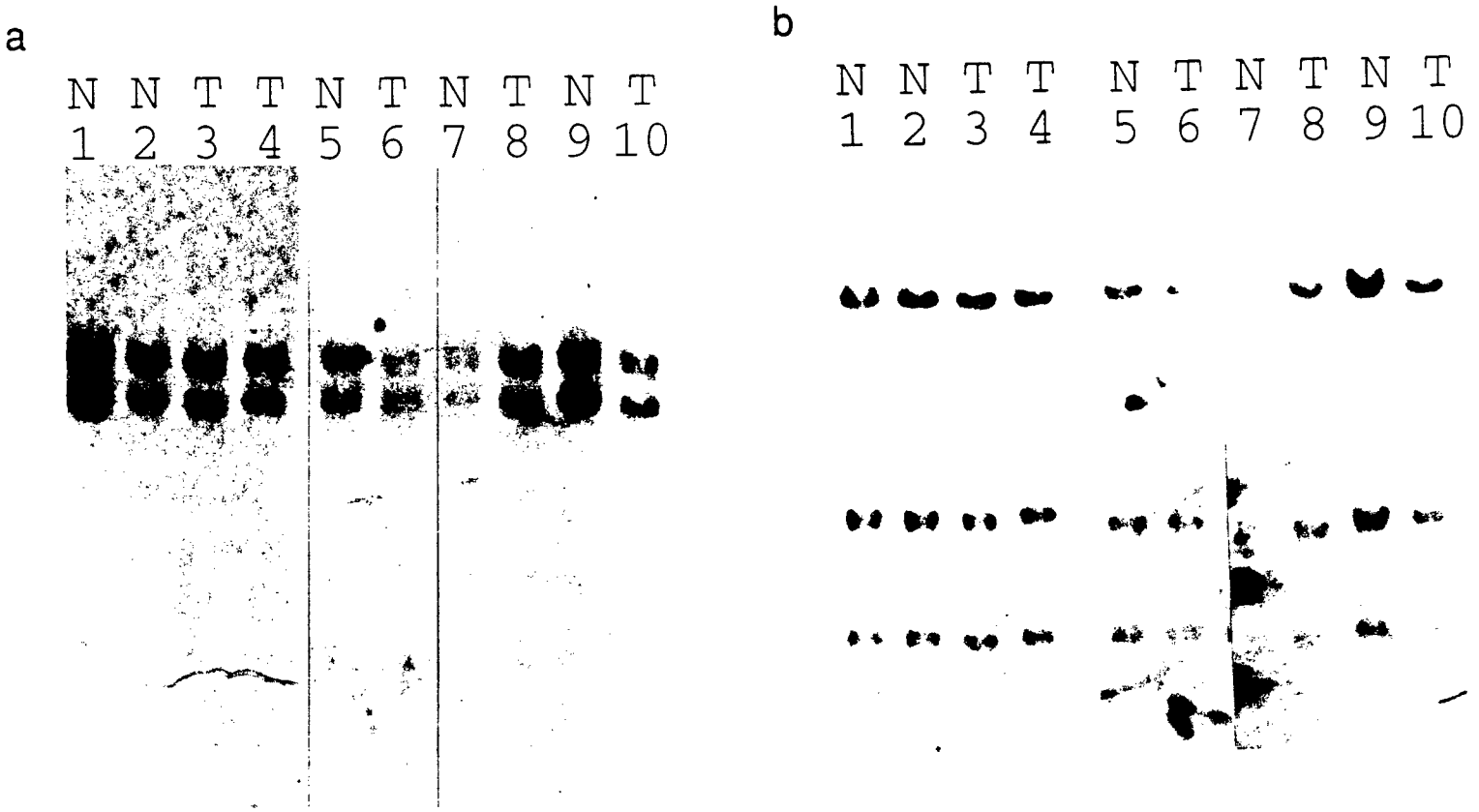

Figure 4. Lack of Intragenic Deletion of TSG101 Gene in Human Breast Cancers. Genomic DNAs were digested with **(a)** Bgl II or **(b)** Pst I, and the filters were probed with the full length cDNA of TSG101. N indicates normal breast tissue DNA and T indicates human primary breast cancer DNA. Lanes 1 and 2, two unrelated normal DNAs; lane 3, tumor 1; lane 4, tumor 3; lanes 5 and 6, normal and tumor of case 2; lanes 7 and 8, normal and tumor of case 5; lanes 9 and 10, normal and tumor of case 6, which did not show a truncated transcript. The Bgl II fragments are 8.5 kb and 6.5 kb, and the Pst I fragments are 13 kb, 4.5 kb, and 2.0 kb.

We then analyzed RNA from breast cancers for evidence of truncated transcripts. Half of breast cancers did contain truncated transcripts as detected by reverse transcription (RT)-PCR (Fig 5).

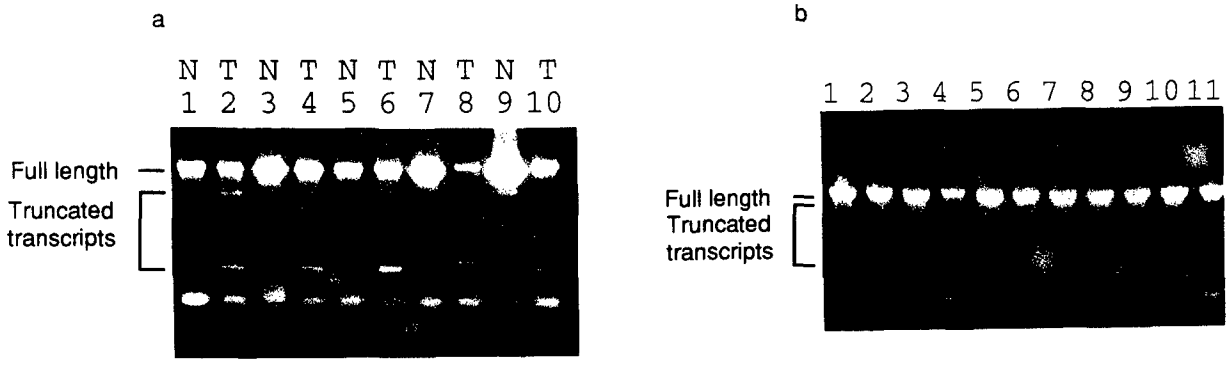

Figure 5. RT-PCR Analysis of Aberrant Transcripts of TSG101. a) RT-PCR analysis of five breast cancer RNAs and their matched normal RNAs. N and T indicate normal and paired tumor RNAs. Lanes 2, 4, 6, 8, and 10 are from tumors 1, 2, 3, 4, and 5; lanes 1, 3, 5, 7, and 9 are from their matched normal tissues, respectively. **b)** RT-PCR analysis of aberrant transcripts in various tissues of two fetuses. Lanes 1 to 6 are from heart, gut, trachea, lung, tongue, and skin of fetus 1; lanes 7 to 11 are from heart, lung, kidney, testes, and brain of fetus 2.

Sequence analysis confirmed that these truncated transcripts contained internal deletions. These sequences are displayed in Figure 6. As shown in Figure 6, the deletions involved at their ends canonical splicing donor and acceptor sites, suggesting that the truncated transcripts were generated by aberrant splicing at pseudosplicing sites.

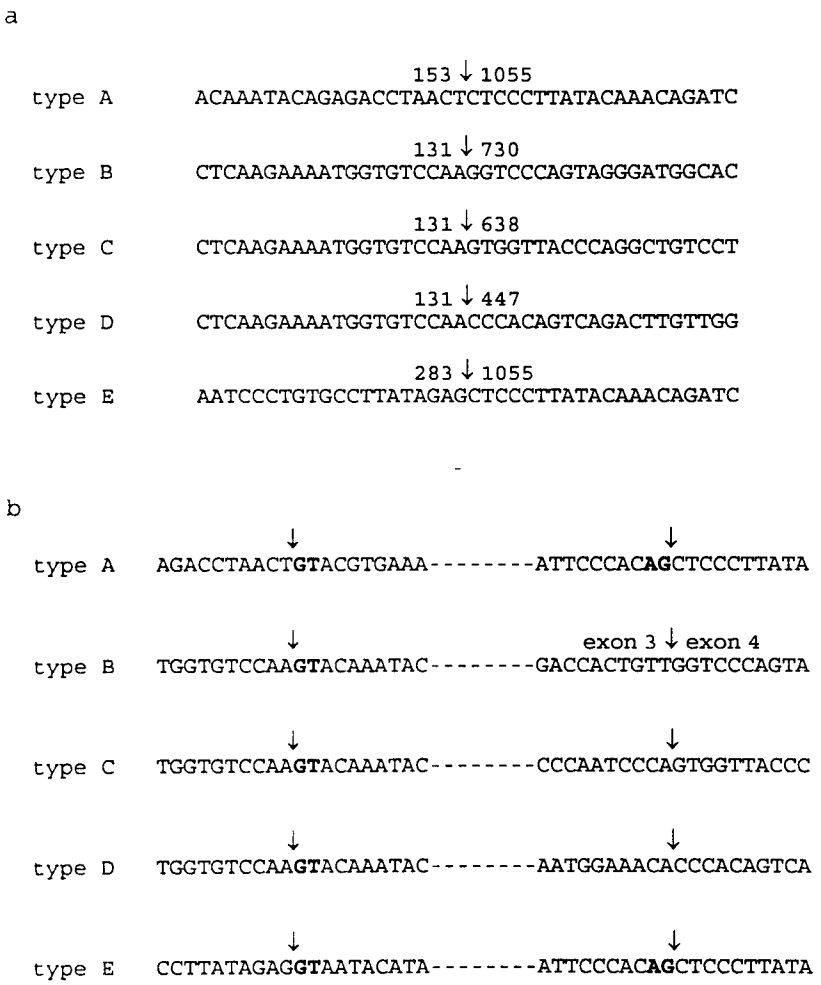

Figure 6. Sequencing Analysis of Aberrant RNA Splicing. **a)** 20 nucleotides on both sides of each deletion type are displayed. Arrows indicate boundaries of deletions. The numbers on the left and right sides of the arrow mark the two nucleotides flanking the deleted region, and these flanking nucleotides remain within the truncated transcripts. **b)** Arrows indicate splicing junctions. Sequences between the arrows are apliced out. The dashed lines indicate nucleotides within the deleted sequences. Boldface highlights canonical splicing donor and acceptor sites. Although there is not a canonical splice acceptor site for the type B truncated transcript, it occurs at the boundary of exons 3 and 4.

For example, the truncated transcript we denote as Type B clearly shows that these transcripts are generated by aberrant splicing, as it uses a GT within exon 1 as the splicing donor, and an intrinsic AG in intron 3 is the splicing acceptor site. Furthermore, because the junction in type A occurs within the interior of exons, and the type A truncated transcript was observed in 4 different tumors, this deletion is very unlikely to be caused by somatic DNA deletion. Rather, our data strongly support the hypothesis that these truncated transcripts are caused by aberrant splicing. Further support for this idea derived from our analysis of normal breast tissue and fetal tissues. Normal tissues showed a small amount of aberrant transcript type A, but none of the other transcripts. Fetal tissue also showed many of the aberrant transcripts seen in normal tissues, but at very low abundance, 1-5% of total RT-PCR products, compared to 50% of RT-PCR products in the tumors. Thus, the aberrant splicing we observe appears to be a property of normal tissue, although it is considerably enhanced in tumors, accounting for a much larger number of transcripts and at greater abundance than the aberrant transcripts seen normally. Finally, to prove that aberrant splicing was caused by a global relaxation of splicing fidelity, and not by mutations in the target genes themselves, we performed full length sequencing analysis of the TSG101 gene in 10 separate breast cancer specimens. None of the tumors showed any mutations within the coding sequence or nearly intron sequences of the gene.

In summary, relaxation of RNA splicing fidelity appears to be a novel form of oncodevelopmental marker for cancer, and it may contribute significantly to the abnormal gene expression that underlies many of the phenotypic properties of tumor cells (22,23).

3. DISCUSSION

Over the past year, we have confirmed from a large number of specimens that breast tumors show maximal LOH centered at D11S860, and extending from D11S988 to D11S1318. This boundary has been confirmed within individual tumors that retain heterozygosity at the centromeric or telomeric ends of this region. These data do not exclude a second more telomeric gene. We have also made a great deal of progress in identifying candidate breast cancer suppressor genes within this region. In addition to NUP98, which we identified earlier, we have identified two additional candidate genes within this interval, that by their sequence analysis are attractive candidates for a role in breast cancer. One of these, which we term TSSC1, is a homologue of the RB associated protein 48, as well as the important chromatin remodeling factor, chromatin assembly factor I. The other, which we term TSSC3, is the human homologue of the mouse gene TDAG51 gene, which activates fas and fasL-mediated apoptosis, an important pathway in breast cancer.

The genetic complementation experiments have continued to frustrate us, in that while we have overcome the problem of culturing these cells, we have observed spontaneous reversion to tumorigenicity of MCF-7 cells containing subchromosomal transferable fragment 74-1-6. We have had similar problems with the cell line G401, and we know of other laboratories that have found similar reversion of tumor suppressor activity using an entire monochromosome 11 in G401 cells. We are therefore repeating these experiments using subchromosomal transferable fragment 74-2, as we have not seen the same problem with reversion in G401 cells. We thus

believe that these experiments will be productive in the coming year, and we look forward to their conclusion.

We have also made a novel observation in breast cancer, not anticipated in the original application, namely the discovery of aberrant splicing of genes in the absence of mutations in the sequence of the gene itself. This aberrant splicing is likely caused by a defect in regulation of the normal splicing machinery, and we believe it can affect a large number of genes generally. Thus, while TSG101 does not map to the critical region containing a tumor suppressor gene on chromosomal 11, other genes that are involved in breast cancer could be affected by this mechanism. Consistent with this hypothesis, we have recently found relaxation of splicing fidelity in NUP98 at very high frequency (> 50%) of the tumors examined to date. Given a potential role of this gene in nuclear transport (15), and an observed defect in nuclear transport of BRCA1 in breast cancer (16), we believe it possible that aberrant splicing of NUP98 could be involved in the pathogenesis of breast cancer.

CONCLUSIONS

1. Breast cancers show frequent LOH centered at D11S860, and LOH extends from D11S988 to D11S1318. These data do not exclude a second more telomeric gene.
2. We mapped to this region the gene NUP98, which is translocated in t(7p15;11p15) in AML, which involves fusion to HoxA9. NUP98 showed a rare sequence variation in the germline of a breast cancer patient, that was not seen in 200 normal control patients.
3. A second gene we identified within the region of LOH, which we denote TSSC1, is a novel homologue of Rb-associated protein 48 (17) and Drosophila chromatin assembly factor (18). The sequence of this gene makes it an attractive candidate for analysis of mutations in breast cancer, analysis of which is in progress.
4. A third gene we identified within this region, which we denote TSSC3, is a novel gene that is the human homologue of mouse TDAG51, which activates fas and fasL-mediated apoptosis. We are investigating a potential role for this gene in breast cancer, given the known defect in this pathway in breast cancer cell lines (20).
5. We have had to overcome additional technical problems using subchromosomal transferable fragment 74-1-6 for analysis of their tumor-suppressing activity in breast cancer. However, we believe that the use of the alternative STF, 74-2, which does not show spontaneous reversion to tumorigenicity in G401 cells, will be successful in these experiments in the coming year, and they are also in progress.
6. We identified a novel mechanism by which breast cancers generate truncated transcripts, namely aberrant splicing in the absence of mutations in the target genes themselves. We showed that TSG101 undergoes frequent aberrant splicing but not mutation in breast cancer. Aberrant splicing of other genes mayalso involve aberrant splicing, and this represents a novel mechanism for tumor progression. This is an exciting observation which we would like to pursue in the coming year as a new task #7. We plan to analyze BRCA1, BRCA2, PTEN, and ER for the presence or absence of aberrant splicing in breast cancers. We have chosen these genes because BRCA1 and BRCA2 show mutations in familial breast cancer, but these mutations do not contribute significantly to sporadic breast cancer. Thus,

observation of abnormal gene expression of these genes due to aberrant splicing would help to define a role for them in nonfamilial disease. We have also chosen PTEN for analysis, because it is a newly discovered tumor suppressor gene and it is frequently mutated in glioblastomas and prostate cancers (24). However, while it is involved in breast cancer, it is mutated in only 6% of cases (24). We thus wish to determine whether aberrant splicing of PTEN contributes to breast cancer more commonly through this novel mechanism of aberrant splicing. Finally, the ER gene often shows reduced expression in breast cancer, which is associated with hypermethylation of CpG islands in the ER gene (25). We wish to determine whether aberrant splicing of ER may also contribute to the reduction of estrogen receptor expression of breast cancer. The studies in this task will not require additional funding in the coming year. However, if they are successful and we can document a role for aberrant splicing in genes that are clearly involved in breast cancer, then we would wish to pursue additional research support from DOD or NIH to measure quantitatively the amount of aberrant transcripts in breast cancer by RNase protection assay and Northern hybridization, and to attempt to identify a functional role for the aberrant transcripts, by genetic complementation experiments or assays for dominant negative activity of the truncated transcripts.

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Advances in Brief

Aberrant Splicing but not Mutations of *TSG101* in Human Breast Cancer[1]

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Abstract

The 11p15 gene *TSG101* was recently reported to undergo frequent large intragenic deletions in human breast cancer. Here we show that that is generally not the case, but the gene shows aberrant splicing, based on the following observations: identical products were observed in matching normal and fetal tissues; deleted cDNA sequence revealed canonical splicing donor and acceptor site sequences; and genomic Southern blots showed no intragenic deletions in all 72 tumors studied. Nevertheless, relaxation of RNA splicing fidelity may be an oncodevelopmental marker in cancer and may play a general role in other genes and tumors.

Introduction

The mouse *TSG101* gene was initially isolated by random homozygous knock out, selecting for inactivated tumor suppressor genes using a NIH3T3 cell transformation assay (1). The human homologue of the *TSG101* gene was then isolated and mapped to 11p15 (2). We had earlier discovered frequent LOH[3] of 11p15 in Wilms' tumor (3), and this observation has been extended to many other cancers, including breast, lung, bladder, testicular, adrenal cortical carcinoma, hepatoblastoma, and ovarian cancer (4–13). We also demonstrated directly the existence of a tumor suppressor gene in this region, using STFs from 11p15, localizing this gene more precisely to a region between the markers *D11S988* and *D11S1318* (14). A second 11p15 tumor suppressor gene may also lie more centromerically between the markers *D11S12* and *L163* (14).[4] Identifying mutations of genes in this region has been a major effort of many laboratories.

Recently, Li *et al.* (2) reported the presence of large intragenic deletions of the *TSG101* gene in 7 of 15 primary human breast cancers, based on the presence of truncated transcripts observed by RT-PCR and Southern hybridization of PCR products of genomic DNA. Remarkably, these deletions involved in many cases the same precise nucleotides, and in some cases, associated intragenic insertions in DNA of the same tumors. They also mapped *TSG101* near the *SAA* gene on 11p15.1-p15.2 (2). We have analyzed *TSG101* in 72 primary breast cancers, at the level of the cDNA by RT-PCR, and at the level of genomic DNA by Southern hybridization. We have also sequenced the full-length cDNA in 10 tumors.

Materials and Methods

Isolation of DNA and RNA from Tissues. Breast cancers and their matched normal tissues were obtained from the Cooperative Human Tissue Network, and normal fetal tissues were from the University of Washington Fetal Tissue Bank. The tissues were stored at −135°C until use. Breast cancers were stages 2–4. The tissues were pulverized in liquid nitrogen and suspended in TE9 [0.5 M Tris-HCl (pH 9.0), 20 mM EDTA (pH 8.0), and 10 mM NaCl]. Proteinase K (0.2 mg/ml) and 1% SDS were added to lyse the cells and digest the proteins at 50°C for overnight. To isolate RNA, tissues were cut into small pieces and homogenized in 4 ml of RNAzol[B] (Tel-Test, Inc.). RNAs were stored at −70°C.

RT-PCR and Sequencing of cDNA. Two μg of RNA were mixed with 50 ng of primer 1 in 35 μl of diethyl pyrocarbonate water, and denatured at 70°C for 10 min; the reverse transcription reaction was carried out in 50 μl solution containing 1× buffer [10 mM Tris (pH 8.0), 50 mM KCl, and 1.5 mM MgCl], 0.2 mM deoxynucleotide triphosphate, 1 unit/μl RNase inhibitor, and 3 units AMV reverse transcriptase. The reaction mixture was incubated at 42°C for 1 h, and cDNA was stored at −20°C. Nested RT-PCR reactions were carried out using the condition of Li *et al.* (2). The RT-PCR products were analyzed in 1.2% agarose gels. DNA fragments were cut out and purified with Qiaex II (Qiagen). DNAs were directly sequenced using an ABI377 automatic sequencer. To quantify the PCR products, we labeled primer 4 with T4 polynucleotide kinase and used 5′ end-labeled primer 4 in the PCR reaction. Agarose gels were dried and analyzed by a PhosphorImager (Molecular Dynamics). The primers used for PCR and sequencing were as follows: primer 1, ATTTAGCAGTCCCAACATTCAGCACAAA; primer 2, CGGGTGTCGGAGAGCCAGCTCAAGAAA; primer 3, CCTCCAGCTGGTATCAGAGAAGTCGT; primer 4, AGCCAGCTCAAGAAAATGGTGTCCAAG; primer 5, TCACTGAGACCGGCAGTCTTTCTTGCTT; primer 6, TTGTCACTGACCGCAGAG; primer 7, ATAGGATGCCGAAATAGG; and primer 8, CCATTCATGTAGATAAGG.

Southern Hybridization. Five μg of genomic DNA were digested with 10 units of restriction enzyme at 37°C overnight. Digested DNAs were resolved on 0.8% agarose gel, transferred to Hybond-N$^+$ filters, and fixed by UV cross-linking. Filters were hybridized with the human *TSG101* cDNA probe prepared by the random priming method (15). Hybridizations were carried out at 65°C overnight in Church-Gilbert buffer [0.5 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM EDTA].

Results

No Intragenic Deletion of *TSG101* in Human Breast Cancer. The *TSG101* gene was recently mapped to 11p15.1-p15.2 and was reported to contain large (7.4–16.4 kb) intragenic DNA deletions in 7 of 15 primary human breast cancers (2). We first mapped the *TSG101* gene using somatic hybrid cells and STFs containing a functional 11p15 tumor suppressor gene(s) (14). *TSG101* mapped centromeric to tumor-suppressing STFs (data not shown). It also mapped at least 6 Mb centromeric to *D11S860,* which is located in 11p15.5 within these STFs and shows the highest frequency of 11p LOH in breast cancer (5).[4] Thus, the *TSG101* gene mapped outside both regions known to contain a tumor suppressor gene.

To assess the frequency of intragenic DNA deletions of *TSG101* gene in human breast cancer, we performed genomic Southern blots on 72 primary tumors probed with *TSG101* cDNA. Genomic DNA was digested with *Bgl*II, which generates 8.5- and 6.5-kb fragments (Fig. 1*a*), and *Pst*I, which generates 13-, 4.5-, and 2.0-kb fragments (Fig. 1*b*). There were no alterations of DNA in any breast cancers (Fig. 1 and Table 1). The digestion pattern was identical for all tumors, matched normal tissues, and unrelated normal tissues. We repeated genomic Southern hybridizations with additional restriction enzyme digests: *Pvu*II (8.5, 5.5, 4.0, 2.5, and 2.0 kb); *Eco*RI (23, 6.5,

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[3] The abbreviations used are: LOH, loss of heterozygosity; STF, subchromosomal transferable fragment; RT-PCR, reverse transcription-PCR.

[4] Unpublished results.

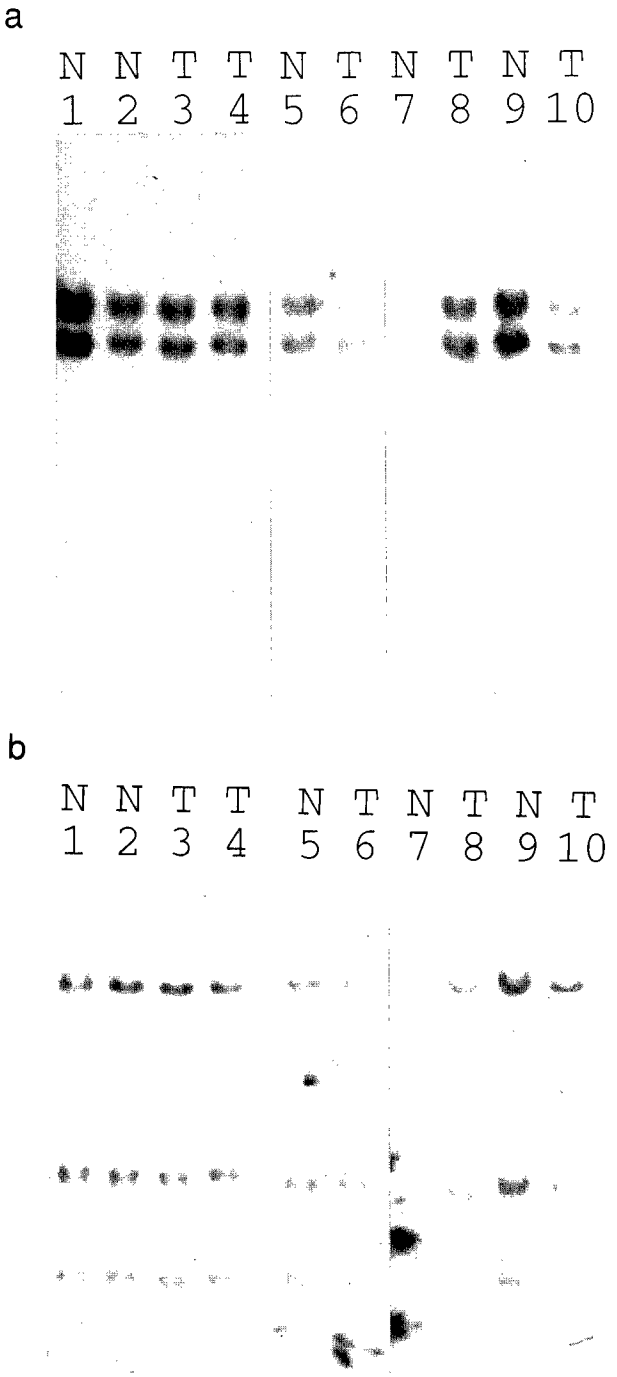

Fig. 1. Lack of intragenic deletion of the *TSG101* gene in human breast cancers. Genomic DNAs were digested with (*a*) *Bgl*II or (*b*) *Pst*I, and the filters were probed with the full-length cDNA of *TSG101*. *N*, normal breast tissue DNA; *T*, human primary breast cancer DNA. *Lanes 1* and *2*, two unrelated normal DNAs; *Lane 3*, tumor 1; *Lane 4*, tumor 3; *Lanes 5* and *6*, normal and tumor of case 2; *Lanes 7* and *8*, normal and tumor of case 5; *Lanes 9* and *10*, normal and tumor of case 6, which did not show a truncated transcript. The *Bgl*II fragments are 8.5 and 6.5 kb, and the *Pst*I fragments are 13, 4.5, and 2.0 kb.

6.0, and 4.5 kb); *Bam*HI (23 kb); and *Sal*I (25 kb). There were no alterations in any tumors (Table 1). For 22 of the breast cancers, matched normal breast tissues were available. DNA samples from the normal tissues showed identical patterns to those of the tumors, as did 10 additional unrelated normal tissues (Fig. 1 and Table 1). The lack of any alteration of DNA fragment on direct genomic Southern blots in any of the 72 breast cancer DNA samples, including those with truncated transcripts, indicated that the truncated transcripts were not caused by intragenic deletions of sufficient size to account for them.

Truncated Transcripts of *TSG101* Caused by Aberrant Splicing. Because there were no intragenic DNA deletions of *TSG101* gene in all 72 breast cancer DNAs analyzed by genomic Southern hybridization, we examined whether there were truncated transcripts as reported by Li *et al.* (2). Five of 12 breast cancers contained truncated transcripts using RT-PCR (*Lanes 2, 4, 6, 8,* and *10* in Fig. 2*a,* corresponding to tumor numbers 1–5). Sequence analysis also confirmed that the truncated transcripts contained internal deletions in the cDNA. Tumor 1 displayed four truncated transcripts in addition to the full-length transcript (Fig. 2*a, Lane 2*), the largest of which we termed type A, which deleted nucleotides from 154 to 1054 (Fig. 3*a* and Table 2). The second largest deletion, termed type B, deleted nucleotides from 132 to 729 (Fig. 3*a* and Table 2). The third largest deletion (type C) deleted nucleotides from 132 to 637 (Fig. 3*a* and Table 2). The smallest deletion, type D, removed nucleotides from 132 to 446 (Fig. 3*a* and Table 2). However, Southern blot analysis of tumor 1 clearly demonstrated no alteration of genomic DNA that could account for the various sized transcripts (Fig. 1, *Lane 3*).

It seems implausible that four cDNA deletions plus a full-length transcript could be generated from intragenic DNA deletions of two chromosomes. We considered that the most likely explanation for the generation of multiple truncated transcripts without apparent DNA deletion was aberrant RNA splicing. Analysis of additional tumors also supported this view. Tumor 2 shows two truncated transcripts in addition to the full-length transcript (Fig. 2*a, Lane 4*). Sequencing analysis of cDNAs from this tumor showed that they contained the type A and type D deletions (Fig. 3*a* and Table 2). Genomic Southern analysis of tumor 2 DNA also showed no deletion that could account for the altered transcript size (Fig. 1, *Lane 6*; matched normal DNA in Fig. 1, *Lane 5*). Tumor 3 showed four truncated transcripts in addition to the full-length transcript (Fig. 2*a, Lane 6*). The second largest deletion represented an additional truncated transcript, termed type F. Sequencing analysis confirmed the presence of types A, B, and D in tumor 3 (Fig. 3*a* and Table 2). Genomic Southern analysis of tumor 3 similarly showed no intragenic deletion (Fig. 1, *Lane 4*). Tumor 4 also showed a truncated transcript as well as the full-length transcript (Fig. 2*a, Lane 8*). The shortened cDNA was a new type E, which deleted nucleotides from 285 to 1054 (Fig. 3*a* and Table 2). Tumor 5 showed two truncated transcripts, types A and D, which were confirmed by sequencing analysis (Fig. 3*a* and Table 2). Genomic Southern analysis of tumor 5 and its matched normal DNA also exhibited no deletion (Fig. 1, *Lanes 7* and *8*). It is clear that although all of these tumors had multiple truncated transcripts, there were no intragenic DNA deletions that could account for them.

Sequence analysis of these transcripts at the junctions of the deleted sequence supported the idea that these truncated transcripts were generated by the aberrant RNA splicing, rather than intragenic DNA deletions. The most common truncated transcript was type A, the deleted sequences of which contained GT at the 5′ end and AG at the 3′ end (Fig. 3*b*), which are canonical splicing donor and acceptor site sequences. Thus, $G_{154}T_{155}$ in exon 1 and $A_{1053}G_{1054}$ in exon 5 were apparently recognized by the RNA splicing machinery. Similarly, the

Table 1 *Genomic Southern analysis of TSG101 gene in human breast cancers*

Seventy-two tumors were analyzed, of which 22 of 72 had matched normal samples. Twenty-one of 72 were tested by at least two different enzyme digests. DNA fragment sizes are in kilobases. There is an *Eco*RI polymorphic site giving an 8.0-kb band in 4 of 33 samples, which was also present in matched normal samples.

Enzymes	Tumors	Matched normals
*Pvu*II (8.5/5.5/4.0/2.5/2.0)	11	11
*Eco*RI (23/6.4/6.0/4.5)	33	8
*Bgl*II (8.5/6.5)	38	6
*Pst*I (13/4.5/2.0)	7	6
*Bam*HI (23)	11	9
*Sal*I (25)	3	2

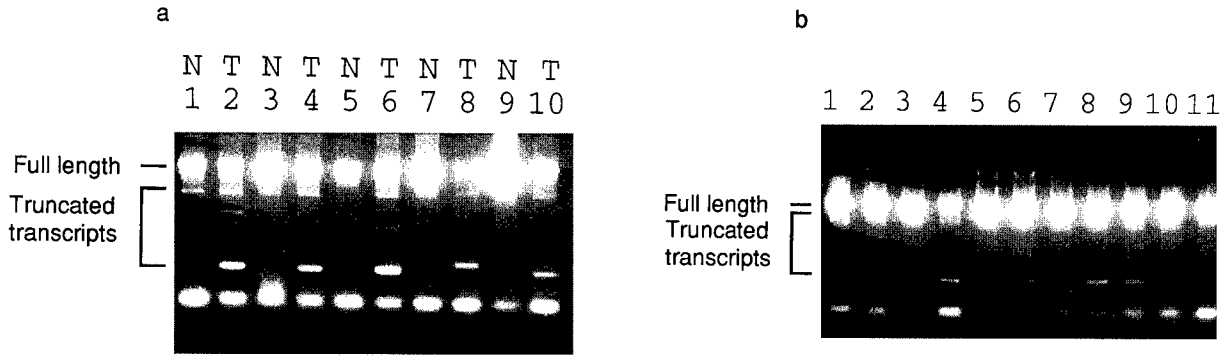

Fig. 2. RT-PCR Analysis of aberrant transcripts of *TSG101*. *a*, RT-PCR analysis of five breast cancer RNAs and their matched normal RNAs. *N* and *T*, normal and paired tumor RNAs, respectively. *Lanes 2, 4, 6, 8,* and *10* are from tumors 1–5, respectively; *Lanes 1, 3, 5, 7,* and *9* are from their matched normal tissues, respectively. *b*, RT-PCR analysis of aberrant transcripts in various tissues of two fetuses. *Lanes 1–6* are from heart, gut, trachea, lung, tongue, and skin of fetus 1; *Lanes 7–11* are from heart, lung, kidney, testes, and brain of fetus 2.

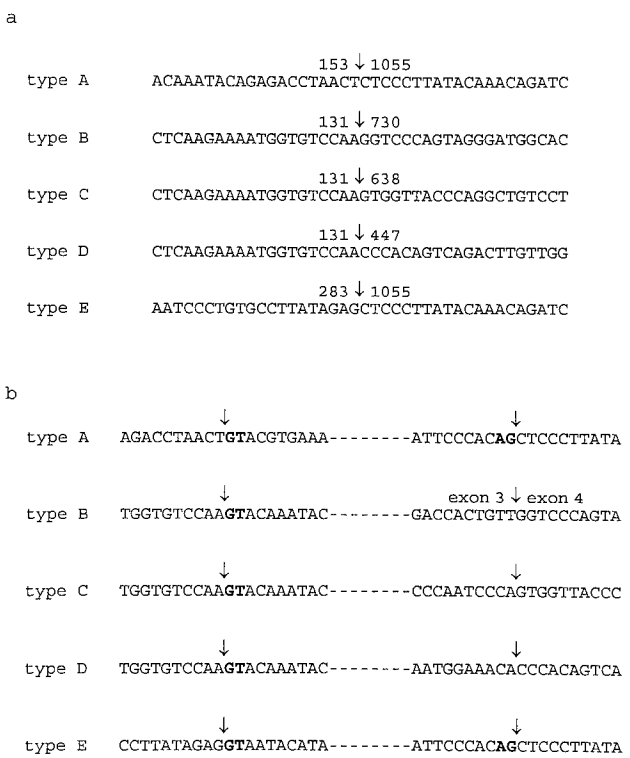

Fig. 3. Sequencing Analysis of Aberrant RNA Splicing. *a*, 20 nucleotides on both sides of each deletion type are displayed. *Arrows*, boundaries of deletions. The numbers on the *left* and *right* sides of the *arrow* mark the two nucleotides flanking the deleted region, and these flanking nucleotides remain within the truncated transcripts. See Table 2 for details. *b*, *arrows*, splicing junctions. Sequences between the arrows are spliced out. *Dashed lines*, nucleotides within the deleted sequences. *Boldface* highlights canonical splicing donor and acceptor sites. Although there is not a canonical splice acceptor site for the type B truncated transcript, it occurs at the boundary of exons 3 and 4.

type E truncated transcript contained GT at the 5′ end and AG at the 3′ end of the deleted sequences (Fig. 3*b*). In this case, $G_{284}T_{285}$ in exon 1 and the same $A_{1053}G_{1054}$ in exon 5 were apparently recognized as splicing sites. The type B truncated transcript was particularly interesting. It used $G_{132}T_{133}$ in exon 1 as the splicing donor and an intrinsic AG in intron 3 as the splicing acceptor site (Fig. 3*b*). Because the junction in type A occurred within the interior of exons and the type A truncated transcript was observed in four different tumors, such precise deletion was very unlikely to be caused by somatic DNA deletion events in different tumors. This again supported the view that these truncated transcripts were generated by aberrant RNA splicing.

Aberrant Splicing in Normal Breast Tissue and Fetal Development. Two observations indicated that the aberrant RNA splicing was not unique to cancer: (*a*) the type D truncated transcript was also present in three of five matched normal samples of these tumors (Fig. 2, *Lanes 1, 3,* and *9*; Table 2), indicating that such splicing can also occur in normal tissue; and (*b*) we found the same aberrant splicing patterns in several fetal tissues from two independent fetuses (Fig. 1*b*). We detected the type A truncated transcript in the heart, gut, lung, tongue, and skin of fetus 1 (Fig. 2*b*, *Lanes 1, 2, 4, 5,* and *6*; Table 2) and both lung and kidney of fetus 2 (Fig. 2*b*, *Lanes 8* and *9*; Table 2). In addition, the gut of fetus 1 contained a type D truncated transcript (Fig. 2*b*, *Lane 2,* and Table 2), the kidney of fetus 2 contained type B and D truncated transcripts (Fig. 2*b*, *Lane 9*), and the brain of fetus 2 contained the type D truncated transcript (Fig. 2*b*, *Lane 11,* and Table 2). The presence of these truncated transcripts in normal fetuses unrelated to tumors clearly demonstrated that these truncated transcripts were not generated specifically in tumors by an intragenic DNA deletion mechanism.

Table 2 *Truncated transcripts of TSG101 in breast cancers and normal tissues*

Cases	RT-PCR[a]	Type of truncated transcripts[b]	Deletions
1T	1	A	154–1054
	2	B	132–729
	3	C	132–637
	4	D	132–446
1N	4	D	132–446
2T	1	A	154–1054
	2	D	132–446
2N	2	D	132–446
3T	1	A	154–1054
	2	F	
	3	B	132–729
	4	D	132–446
4T	1	E	285–1054
5T	1	A	154–1054
	2	D	132–446
5N	2	D	132–446
Fetus 1			
Heart	1	A	154–1054
Gut	1	A	154–1054
	2	D	132–446
Lung	1	A	154–1054
Tongue	1	A	154–1054
Skin	1	A	154–1054
Fetus 2			
Lung	1	A	154–1054
Kidney	1	A	154–1054
	2	B	132–729
	3	D	132–446
Brain	1	D	132–446

[a] The RT-PCR products were labeled from small to large fragment sizes as 1–4. The fragment in the matched normal tissue (N) was assigned to the same number as the corresponding fragment in the tumor (T).

[b] Many of the RT-PCR fragments were sequenced. Some were deduced based on the size. Also see Fig. 3 for the sequences flanking the splicing junction.

There did appear to be a genuine increase in the number of classes of aberrant transcripts in tumors compared to normal breast tissue. Except for type D, none of the other truncated transcripts was detected in the matched normal breast tissue samples (Fig. 2*a* and Table 2). Comparison of normal and tumor of case 1 revealed the presence of a comparable amount of type D truncated transcript, whereas truncated transcripts A, B, and C were exclusively the products of the tumor. Similarly, we detected comparable amounts of type D truncated transcript in both normal and tumor of cases 2 and 5, whereas other types of truncated transcripts were detected only in tumors and fetuses (Fig. 2*a* and Table 2). Using 5′-end labeled primers in the PCR, we quantified the amount of truncated transcripts by PhosphorImager analysis. The types A, B, C, and D truncated transcripts accounted for 63, 7, 6, and 9% of RT-PCR products in tumor 1, respectively. In the matched normal tissue, the type D truncated transcript accounted for 8% of the RT-PCR product, whereas types A, B, and C did not appear. Similarly, the truncated transcripts accounted for 85, 63, 59, and 33% (sum of all of the aberrant transcripts) in tumors 2–5, respectively. Matched normal tissue from three of these cases again showed truncated transcript D, accounting for 3–4% of mRNA. The normal fetal tissues showed numbers of truncated transcripts comparable to that of the tumors, but at lower relative levels of expression (types A, B, and D, at 1–5% of total RT-PCR products) in heart, gut, lung, tongue, skin of fetus 1, and lung, kidney, and brain of fetus 2. Therefore, relaxation of splicing fidelity is associated with both tumorigenesis and fetal development.

No Point Mutation of *TSG101* Detected in Breast Cancers. We have shown that there were no intragenic DNA deletions of *TSG101* in breast cancer and that truncated transcripts are caused by aberrant RNA splicing. To determine whether somatic alterations of *TSG101* play any common role in breast cancers, we looked for evidence of subtle changes, such as point mutation or small insertion/deletions of the gene. We amplified the entire coding region of the *TSG101* gene from 10 breast cancers and sequenced all 10 cDNAs. No point mutations or small insertions/deletions were detected (data not shown). We concluded that there were neither point mutations nor intragenic deletions of *TSG101* in the breast cancers studied here.

Discussion

In summary, we have found frequent truncated transcripts of TSG101 in human breast cancer but no intragenic deletions that would account for them in 72 breast cancers studied. We have shown that the truncated transcripts can occur in both matched normal breast tissues and normal fetal tissues as well as breast cancer, and that these truncated transcripts are caused by aberrant RNA splicing. Finally, no mutations were found over the entire coding sequence in 10 tumors.

These results have three important implications:

(*a*) *TSG101* is unlikely to show frequent somatic mutation in human breast cancer, although we cannot exclude rare mutations of the gene. Furthermore, we mapped *TSG101* outside the two known regions of tumor suppressor activity by genetic complementation as well as by LOH studies. Finally, we found no deletions on Southern hybridization of genomic DNA that could account for the shortened cDNA products. We could also not detect such deletions after PCR of DNA using the identical conditions of Li *et al.* (Ref. 2 and data not shown). Thus, *TSG101* is not the 11p15 tumor suppressor gene commonly involved in breast cancer.

(*b*) Aberrant RNA splicing of *TSG101* clearly underlies the abnormal transcripts seen, as evidenced by the presence of identical products in matched normal tissues and normal fetal samples, the presence of four truncated transcripts in individual specimens, the presence of the full-length transcript in all tumor samples, the absence of appropriately sized deletions on genomic Southern blots, and the presence of canonical splicing donor and acceptor site sequences within the deleted DNA.

(*c*) This aberrant splicing does appear to increase in tumors, at least in the number of aberrant transcripts that are seen, and it can also occur in fetal tissues. Thus, relaxation of RNA splicing fidelity appears to be a novel form of oncodevelopmental marker for cancer. Normal embryonic cells possess phenotypic markers common to many cancer cells (*e.g.*, α-fetoprotein, carcinoembryonic antigen, and fetal isoenzyme patterns). Because of these observations, many observers, beginning with Laennec, have proposed that some steps in cancer involve the abnormal expression of normal cellular genes (16, 17). Thus, relaxation of RNA splicing fidelity may contribute generally to abnormal gene expression in cancer. We have recently found that *Staf50* also shows aberrant splicing products in Wilms' tumor and breast cancer.[5] Nevertheless, it is important to consider that aberrant splicing rather than somatic mutation may be present when altered transcripts are observed in tumors.

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[5] Unpublished observations.

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